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# De Novo Loss-of-Function Mutations in *USP9X* Cause a Female-Specific Recognizable Syndrome with Developmental Delay and Congenital Malformations

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Mutations in more than a hundred genes have been reported to cause X-linked recessive intellectual disability (ID) mainly in males. In contrast, the number of identified X-linked genes in which de novo mutations specifically cause ID in females is limited. Here, we report 17 females with de novo loss-of-function mutations in *USP9X*, encoding a highly conserved deubiquitinating enzyme. The females in our study have a specific phenotype that includes ID/developmental delay (DD), characteristic facial features, short stature, and distinct congenital malformations comprising choanal atresia, anal abnormalities, post-axial polydactyly, heart defects, hypomastia, cleft palate/bifid uvula, progressive scoliosis, and structural brain abnormalities. Four females from our cohort were identified by targeted genetic testing because their phenotype was suggestive for *USP9X* mutations. In several females, pigment changes along Blaschko lines and body asymmetry were observed, which is probably related to differential (escape from) X-inactivation between tissues. Expression studies on both mRNA and protein level in affected-female-derived fibroblasts showed significant reduction of *USP9X* level, confirming the loss-of-function effect of the identified mutations. Given that some features of affected females are also reported in known ciliopathy syndromes, we examined the role of *USP9X* in the primary cilium and found that endogenous *USP9X* localizes along the length of the ciliary axoneme, indicating that its loss of function could indeed disrupt cilium-regulated processes. Absence of dysregulated ciliary parameters in affected female-derived fibroblasts, however, points toward spatiotemporal specificity of ciliary *USP9X* (dys-)function.

X-linked intellectual disability (ID) with presumed recessive inheritance pattern is shown to be caused by mutations in more than a hundred genes.<sup>1,2</sup> Most families display a clear X-linked segregation pattern, in which males are affected and females are unaffected or mildly affected carriers.<sup>3–5</sup> In contrast, the number of identified X-linked genes in which de novo mutations cause ID specifically in females is limited.

Using whole-exome sequencing (WES), SNP array, array CGH, and CytoScan HD array in a diagnostic setting as

described before,<sup>6–11</sup> we identified 13 de novo loss-of-function mutations in *USP9X* (Ubiquitin-specific protease 9 [MIM: 300072; GenBank: NM\_001039590.2]) in females with ID/developmental delay (DD) and multiple congenital malformations (Figures 1A and 1B; Table S1). Female 7 was previously reported as part of a large study sequencing individuals with ID, congenital anomalies, and/or autism with a targeted gene panel.<sup>9</sup> Written consent was obtained from the legal guardians for all females and the study was given IRB approval. We recognized a

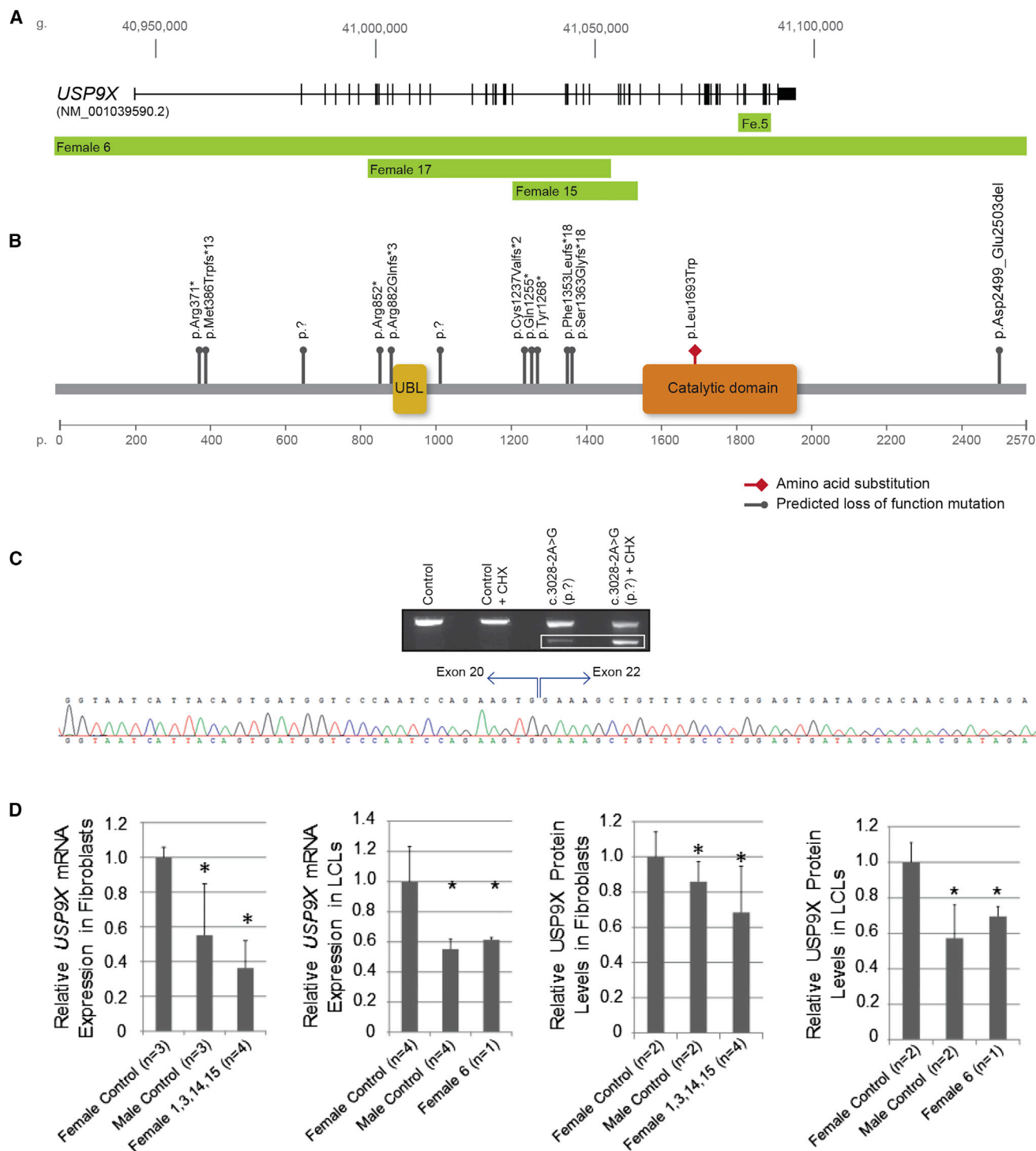
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**Figure 1. Identified De Novo *USP9X* Loss-of-Function Mutations**

(A) Detailed view of the *USP9X* (GenBank: NM\_001039590.2) region and the reported deletions.

(B) Overview of *USP9X* including UBL and catalytic domain and the location of reported mutations according to their relative position at the protein level. The reported amino acid substitution is located within the catalytic domain.

(C) RNA was extracted from both control and affected female (c.3028–2A>G [p.(?)]) fibroblasts cultured under normal conditions or in the presence of cycloheximide (CHX) to inhibit NMD. After cDNA synthesis and PCR, agarose gel analysis showed two different product sizes generated from the c.3028–2A>G transcript but only one from the control fibroblast transcript. Excision and sequencing of the additional band revealed that the aberrant *USP9X* transcript lacked exon 21. The level of the aberrant transcript was increased 4-fold when fibroblasts were treated with cycloheximide, confirming that the aberrant transcript was indeed subjected to NMD.

(legend continued on next page)

similar pattern of facial characteristics, congenital malformations, and brain abnormalities in these females. Four additional affected females were identified because their phenotype was suggestive for *USP9X* mutations. Subsequently, de novo protein-truncating mutations and intra-genic *USP9X* deletions were duly demonstrated by Sanger sequencing, WES, or CytoScan HD array (Figures 1A and 1B; Table S1), illustrating the clinical recognizability of this new syndrome. All females (age ranging 2 years, 7 months to 23 years) with de novo mutations shared a distinct phenotype. They showed mild to moderate ID with motor and language delay, short stature, hearing loss, and distinct congenital malformations, notably choanal atresia, asymmetric hypomastia, cleft palate/bifid uvula, heart defects, progressive scoliosis, post-axial polydactyly, and anal abnormalities (Tables 1 and S2; case studies in Supplemental Note). Shared facial characteristics included prominent forehead, low nasal bridge, prominent nose with flared alae nasi, thin upper lip, smooth and long philtrum, and ears that were low set, posteriorly rotated, and dysplastic (Figure 2A). In addition to the *USP9X* variant, female 5 also harbored a de novo variant in *PTPN11* (MIM: 176876), which has previously been reported to cause Noonan syndrome (MIM: 163950).<sup>12</sup> Though all features that were observed in this female could potentially be explained by the *USP9X* variant itself (Table S2), a contribution of aberrant *PTPN11* to phenotypic features such as intellectual disability, short stature, and heart defect in this female is likely. Neuroimaging reports were available for 13 out of 17 females (Table S2). Detailed evaluation of brain images of five of these females (females 1, 2, 3, 7, and 16) showed asymmetric hypoplasia of the cerebellar vermis and hemisphere with a retrocerebellar cyst, short and thin corpus callosum, thin brainstem, and mildly abnormal frontal gyration pattern (Figure 3). Notably, we observed thyroid hormone abnormalities in six of the females, requiring medical treatment in three of them.

The X-linked *USP9X* encodes a structurally and functionally highly conserved deubiquitinating enzyme, containing a UBL (ubiquitin-like) and a catalytic ubiquitin specific protease (USP) domain.<sup>13–15</sup> It is known to play an important role in neural development of both humans and mice and is required for fetal development.<sup>16–18</sup> *USP9X* is highly expressed during embryogenesis and expression declines as cell fates become restricted.<sup>18</sup> The *USP9X* ortholog in *Drosophila*, fat facets (*faf*), has been shown to be important in cell polarity and cell fate of the developing eye in *Drosophila*.<sup>19</sup> A range of signaling proteins involved in different neurodevelopmental pathways including Notch,

**Table 1. Clinical Features of Females with De Novo *USP9X* Loss-of-Function Mutations**

	Percentage	Number
<b>Development</b>		
Intellectual disability or developmental delay	100%	17/17
<b>Growth</b>		
Short stature	53%	9/17
<b>Congenital Abnormalities</b>		
Eye abnormality	59%	10/17
Choanal atresia	35%	6/17
Cleft palate/bifid uvula	29%	5/17
Dental abnormality	71%	12/17
Asymmetric hypomastia	29%	5/17
Heart defect	44%	7/16
Urogenital abnormality	29%	5/17
Sacral dimple	29%	5/17
Scoliosis	65%	11/17
Hip dysplasia	47%	8/17
Post-axial polydactyly	53%	9/17
Abdominal wall abnormality	12%	2/17
Anal atresia	53%	9/17
<b>Neurology</b>		
Seizures	24%	4/17
Hypotonia	47%	8/17
<b>Brain Abnormalities</b>		
Dandy walker malformation (variant)	38%	5/13
Hypoplastic corpus callosum	62%	8/13
(Asymmetric) cerebellar hypoplasia	55%	6/11
(Asymmetric) enlarged ventricles	73%	8/11
Thin brain stem	30%	3/10
Abnormal gyration pattern frontal lobe	50%	5/10
<b>Other</b>		
Hearing loss	65%	11/17
(Blaschko) pigment abnormality	65%	11/17
Hypertrichosis	29%	5/17
Leg length discrepancy	41%	7/17
Malignancy	12%	2/17
Recurrent respiratory tract infections	53%	9/17
Thyroid hormone abnormality	35%	6/17

(D) *USP9X* expression is depleted in female cell lines harboring loss-of-function alleles. Relative qPCR analysis of *USP9X* mRNA and relative quantification of immunoblot analysis of *USP9X* protein derived from female and male control cell lines and from affected female cell lines. n = the number of individual cell lines analyzed. Each cell line analyzed in quadruplicate. Error bars represent SDs. Asterisk (\*) indicates significantly different to female controls,  $p < 0.05$  by Student's t test.





**Figure 2. Clinical Characteristics of Females with De Novo *USP9X* Loss-of-Function Mutations**

(A) Frontal and lateral photographs of females with de novo mutations in *USP9X*. Shared facial characteristics include facial asymmetry, prominent forehead, bitemporal narrowing, short palpebral fissures, low nasal bridge, prominent nose with flared alae nasi from adolescence age, thin upper lip, smooth and long philtrum, hanging full cheeks in early childhood, and low-set, posteriorly rotated, and dysplastic ears with attached lobule.

(B) Photographs of the hands of seven affected females. Shared characteristics include ulnar deviation of 5<sup>th</sup> digit, tapered fingers, short 4<sup>th</sup> and 5<sup>th</sup> metacarpals, and post-axial polydactyly (simian crease present but not shown).

(C) Photographs of the feet of five affected females. Shared characteristics include hallux valgus and sandal gap (pes cavus present but not shown).

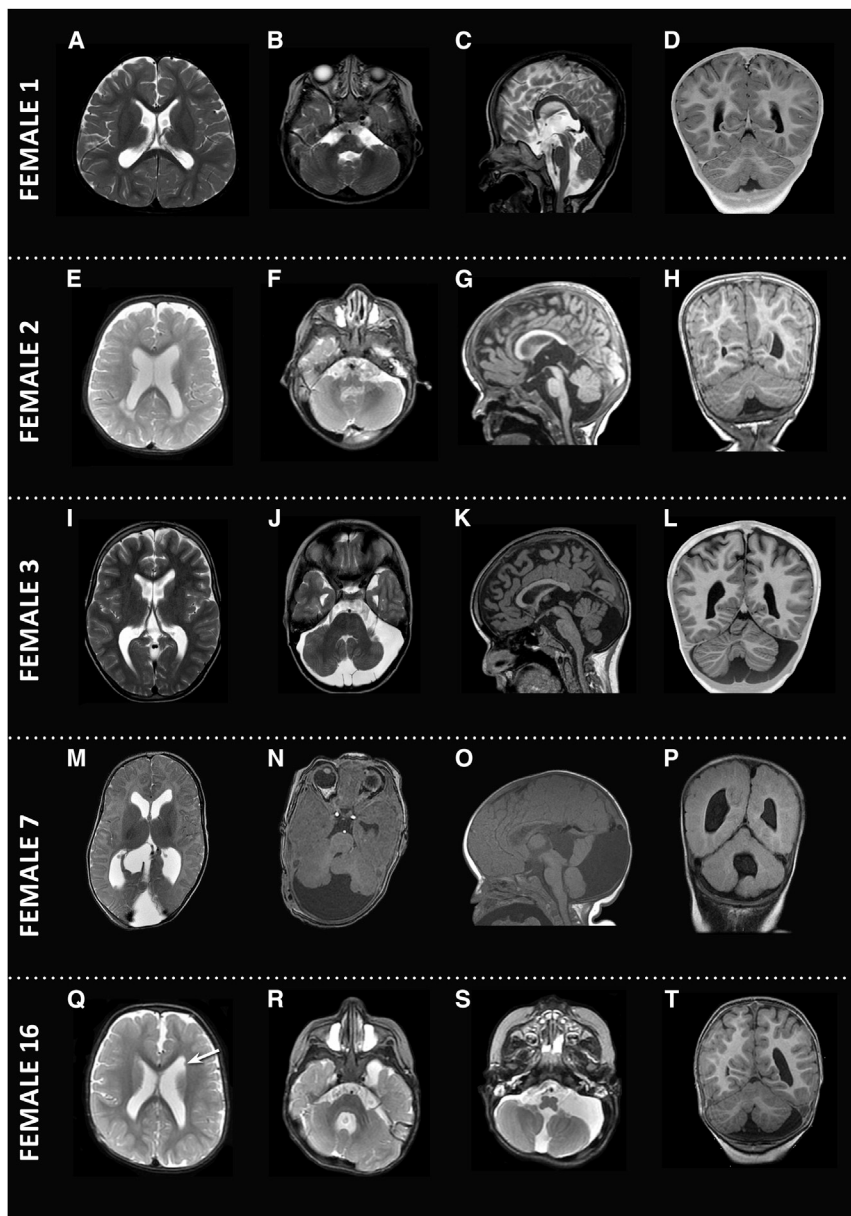
(D) Observed Blaschko lines of female 3, indicative for 11 of the affected females, suggestive of different X-inactivation pattern between tissues (functional mosaicism).

Wnt, TGF- $\beta$ , and mTOR have been shown to interact with *USP9X*.<sup>14,20–27</sup> *USP9X* also has been described to act as both an oncogene and tumor-suppressor gene and is frequently found to be dysregulated in human cancer.<sup>14,28,29</sup> Two of the affected females developed malignancy at a young age (22 and 8 years). Both acute lymphoblastic leukemia and osteosarcoma were treated successfully and have not reoccurred. To determine the risk and nature of particular malignancies in this new syndrome, further studies are required.

We observed pigment abnormalities along Blaschko lines and facial asymmetry, asymmetric abnormalities of the brain and breast, and asymmetric length of the legs (Figures 2A, 2D, and 3), all suggestive of a pattern of post-zygotic mosaicism or differential X-inactivation (XCI) between tissues (functional mosaicism).<sup>30</sup> *USP9X* is one of the genes shown to escape XCI.<sup>31,32</sup> However, it is known that most of the genes that escape from XCI are not fully expressed from the inactivated X chromosome and instead show a partial escape.<sup>33–35</sup> Moreover, there is accumulating evidence for tissue-specific and developmental-stage-dependent differences in XCI and variability of escape of *USP9X*.<sup>34,36–39</sup> In the partial escaping genes, non-random XCI or skewing, as observed often in female carriers of an X-linked mutation, will only partially restore a normal phenotype.<sup>35</sup> Consistent with this hypothesis, XCI was found to be skewed >90% in fibroblasts in three

of the five of the tested females, but skewing was not related to disease severity (Table S3). We note that a similar skewing pattern of XCI was observed recently in females with de novo mutations in *DDX3X* (MIM: 300160), another X-chromosomal gene that has been suggested to escape XCI and in which de novo mutations cause ID specifically in females.<sup>40</sup>

In one of the affected females, a predicted splice site mutation was identified. To evaluate whether this mutation indeed results in an aberrant transcript, we synthesized cDNA from RNA extracted from primary skin fibroblasts of both the affected female and a control. We amplified a fragment of 576 base pairs (bp) covering exon 20 to exon 22 by PCR. Electrophoretic separation showed two products of 576 and 455 bp in the sample from the affected female, and a single 576-bp product in the control. Sequencing of the smaller product revealed that this cDNA transcript from the affected female indeed lacked exon 21, confirming the truncating effect of the splice site mutation. Importantly, the level of the transcript was increased 4-fold when fibroblasts were treated with cycloheximide, strongly suggesting that the aberrant transcript was subjected to nonsense-mediated mRNA decay and as such leads to loss of function of this *USP9X* allele (Figure 1C). To study the effect of the heterozygous loss-of-function *USP9X* alleles on their mRNA expression and protein levels, we performed

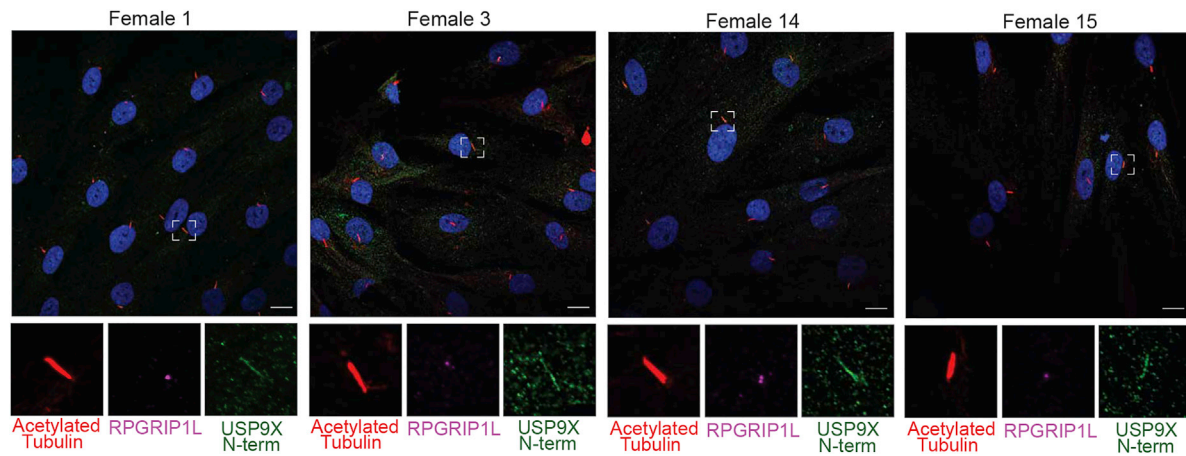


**Figure 3. Representative MRI Images from Females 1, 2, 3, 7, and 16 with De Novo USP9X Loss-of-Function Mutations**  
(A–D) Female 1 (2 years): MRI T2 axial (A, B) and sagittal (C) and T1 axial (D) sections show brachycephaly, mild enlargement of the lateral and 3<sup>rd</sup> ventricles; mild hypoplasia of cerebellar vermis and left cerebellar hemisphere; enlarged IV ventricle and cisterna magna with small retrocerebellar cyst; thin brain stem and mesencephalon; relatively small frontal lobes with somewhat simplified gyration; and short hypoplastic corpus callosum (both rostrum and splenium).  
(E–H) Female 2 (1.5 years): MRI T2 axial (E, F) and T1 sagittal (G) and coronal (H) sections show enlargement of the lateral ventricles, mild hypoplasia of cerebellar vermis and left cerebellar hemisphere; enlarged cisterna magna; thin corpus callosum, pons, mesencephalon, and brain stem; and broader and underdeveloped frontal gyri.  
(I–L) Female 3 (11 years): MRI T2 axial (I, J) and T1 sagittal (K) and axial (L) sections show asymmetric enlargement of the lateral ventricles; simplified convolutions of the frontal lobe gyri; hypoplasia of cerebellar vermis and left hemisphere; large cisterna magna and retrocerebellar cyst; and thin corpus callosum with hypoplasia of the rostrum.  
(M–P) Female 7: MRI T2 axial (M), T1 axial (N), T1 sagittal (O), and coronal FLAIR (P) sections show macrocephaly; enlargement of the lateral and 3<sup>rd</sup> ventricles with an interhemispheric cyst; dysplastic cerebellar hemispheres; dysplasia of the cerebellar vermis which is uplifted, with a high position of the tentorium and a large posterior fossa, typical of Dandy-Walker malformation; and thin and hypoplastic corpus callosum.  
(Q–T) Female 16 (2 years): MRI T2 axial (Q, R, S) and T1 coronal (T) sections show enlarged lateral ventricles; irregular gyri of the cerebral cortex with irregular depth of the sulci in frontal and perisylvian areas;

small heterotopic nodule of gray matter (arrow) and thin and hypoplastic corpus callosum (both rostrum and splenium); hypoplasia of the anterior cerebellar vermis and left cerebellar hemisphere; enlarged cisterna magna and arachnoidal cyst surrounding the cerebellum, especially at the left side; and mild hypoplasia of pons and brain stem. This female was identified with Sanger sequencing based on these brain abnormalities in combination with ID, dysmorphic features, and congenital abnormalities.

both qRT-PCR and immunoblot analysis of fibroblasts ( $n = 4$ ) and lymphoblastoid cell lines (LCLs;  $n = 1$ ) derived from affected females and both female and male controls (Figures 1D and S1). We found that expression of *USP9X* in affected females was reduced compared to control females in both fibroblasts and LCLs at both mRNA expression and protein levels. Although some cellular variability was evident, on average this decrease was significant ( $p < 0.05$  by Student's  $t$  test) (Figures 1D and S1). There was no correlation between skewing of XCI and expression of mRNA and protein level. Whether the cells in affected tissue have benefit from the skewed XCI remains uncertain. The escape from XCI was sup-

ported by the fact that the average expression of *USP9X* mRNA in both control male fibroblasts and control male LCLs was ~50% of that in female controls. After quantification of protein levels in male control LCLs, similar levels were observed. The *USP9X* protein level in male control fibroblasts was increased to ~80% of that in female controls, but was still significantly less than the protein level in female controls. Intriguingly, these data thus reveal that affected females displayed reduced levels of *USP9X* compared to female controls but comparable levels to that in healthy control males. It will be important to expose whether these trends extend to other tissues, where the level of escape from XCI might



**Figure 4. USP9X Localizes to the Primary Cilium**

To induce ciliogenesis, control and affected female fibroblasts, matched for gender and age, were starved for 48 hr prior to immunofluorescence labeling. Endogenous USP9X is detected along the length of the axoneme of primary cilia, using an antibody against its N terminus (N-Term, shown in green) as compared to the ciliary markers RPGRIP1L (pink, denoting the ciliary transition zone at the base of the cilium) and acetylated alpha-tubulin (red, marking the ciliary axoneme). DAPI (blue) stains the nuclei; scale bars represent 10  $\mu$ m. Ciliated fibroblasts derived from affected females are shown here, USP9X localization in control fibroblasts is shown in [Figure S2](#).

not be comparable. Furthermore, characterization of different expression patterns between sexes (described for USP9X in brain thus far<sup>41</sup>) and/or that of protein levels of USP9X substrates will be important to ascertain as well.

In contrast to the severely disruptive de novo mutations in females, three milder mutations in USP9X have been reported in males without multiple congenital malformations. The mutations were transmitted by phenotypically normal females and resulted in ID, hypotonia, and behavioral problems in the males.<sup>42</sup> An additional two missense mutations were identified after resequencing of USP9X in a cohort of 284 males with epilepsy.<sup>43</sup> For two de novo mutations reported in large autism cohorts, no specific gender information was described.<sup>44</sup> The phenotype of the males differs notably from the observed phenotype in the affected females described here. They had ID and short stature, but lacked the multiple congenital malformations observed in affected females. Besides four missense mutations, one frameshift mutation has been reported in the males. This single frameshift mutation occurred within the last 50 nucleotides upstream of the last 3'-exon-exon junction, presumably escaping nonsense-mediated mRNA decay (NMD), and therefore results in a truncated protein lacking the last exon.<sup>45,46</sup> Because no truncating variants have been described in healthy controls in the ExAC database and no mutations causing loss of function of USP9X have been reported in males, we suspect that loss-of-function mutations could be lethal in males. This hypothesis is further supported by the fact that the absence of Usp9X in male mice is embryonically lethal.<sup>47</sup> In contrast, all but one of the affected females we report here have protein-truncating mutations and deletions. In one female, we identified a de novo missense mutation, located in the catalytic

domain of the protein. Given the fact that this female was phenotypically comparable with the other females, it is likely that this specific missense mutation leads to loss of function of the protein. We hypothesize that in addition to complete loss-of-function mutations, such as protein-truncating mutations and deletions, a small subset of specific missense mutations will also lead to disease in females.

Affected females presented with symptoms that overlap with CHARGE syndrome (MIM: 214800) (*CHD7* [MIM: 608892] tested in four of the females) and with the clinical spectrum of some known ciliopathy syndromes, such as Bardet-Biedl, Meckel-Gruber, and Joubert syndromes.<sup>48</sup> Therefore, we investigated whether heterozygous protein-truncating mutations result in the disruption of ciliary structure, formation, or trafficking in fibroblasts of four of the affected females we had available (females 1, 3, 14, and 15). First, we determined subcellular localization of endogenous USP9X in both controls and affected-female-derived fibroblasts under serum starvation to induce ciliogenesis, as well as in serum-rich conditions.<sup>49,50</sup> USP9X showed diffuse cytoplasmic staining with areas enriched with puncta consistent with its described association with protein and vesicle trafficking.<sup>51</sup> Importantly, upon the induction of ciliogenesis in the fibroblasts, USP9X was indeed found to localize to the cilium. This ciliary localization was observed along the length of the ciliary axoneme of most fibroblasts, and comparable in cells from affected females and from age- and gender-matched controls ([Figure 4](#)). This localization was significantly decreased with siRNA knockdown of USP9X indicating specificity of the signal ([Figure S2](#)). We were unable to observe any significant differences in ciliogenesis, ciliary length, or ciliary trafficking between fibroblasts from affected females when compared to controls



(Figure S3), and siRNA knockdown of *USP9X* did not impair ciliogenesis in fibroblasts. This suggests that *USP9X* dosage is not critical to the generation of primary cilia in these fibroblasts, despite localization of *USP9X* in their cilia. It is therefore more likely that *USP9X*-regulated signal transduction pathways mediated by the primary cilium are more subtly disturbed, and/or that this disturbance is spatiotemporally restricted to the tissues affected in this specific phenotype, possibly due to tissue-specific and developmental-stage-dependent differences in XCI and variability of escape of *USP9X*. Future studies utilizing dedicated cell-based or animal models will be necessary to evaluate these mechanisms.

In conclusion, this study defines a recognizable X-linked ID/DD syndrome with associated multiple congenital malformations and brain abnormalities specific to females, caused by de novo loss-of-function mutations in *USP9X*, a gene known to escape X-inactivation. The phenotypic characteristics overlap with ciliopathy conditions and *USP9X* localization along the length of the ciliary axoneme of fibroblasts indicates a role in de-ubiquitination of ciliary proteins, which could contribute to the disease pathogenesis of this specific syndrome.

#### Accession Numbers

The accession numbers of the CNV data reported in this paper are DECIPHER: 322370, 322371, 322372, and 322375.

#### Supplemental Data

Supplemental Data include Supplemental Acknowledgments, case reports in a Supplemental Note, three figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2015.12.015>.

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#### Web Resources

The URLs for data presented herein are as follows:

DECIPHER, <http://decipher.sanger.ac.uk/>

Ensembl Genome Browser, <http://www.ensembl.org/index.html>

ExAC Browser, <http://exac.broadinstitute.org/>

OMIM, <http://www.omim.org/>

RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>

UCSC Genome Browser, <http://genome.ucsc.edu>

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